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New flax producing bioplastic fibers for medical purposes

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ABSTRACT

Flax (*Linum usitatissimum*) is an annual plant with a long history of cultivation and a great significance in medicine and industry. To increase the valuable qualities of flax products, the flax genome has been genetically modified, with the specific aims to improve flax properties and usefulness for various industries. Through introduction of polyhydroxybutyrate (PHB) synthesis genes from *Ralstonia eutropha* into flax genome, biomechanical properties of fiber have been improved. In this paper, we report that those fibers contain higher quantities of phenolics in addition to PHB thus making the modified fibers a very suitable material for biomedical application, provided that the fabric is not treated chemically. The linen PHB-fabric promotes human fibroblast proliferation and has been shown to have antimicrobial activity in the *in vitro* studies. Based on this quality of the fabric, the new dressing for chronic wounds was developed and proven to be successful in a pre-clinical trial. Therefore, it was demonstrated that modified flax fibers are suitable material for biomedical industry.

1. Introduction

For centuries, natural fibers from various species have been used by human society for many applications. Flax, generally regarded as a dual-purpose plant due to its products, the fiber and seed, is one of such plants. Fiber, as a raw material for yarn production, served as a major source to manufacture textiles for clothing and in composite production (construction or automobile industries) (Bledzki et al., 2008; Powell et al., 2002), whereas seeds were pressed to extract edible oil. On the whole, bast flax fiber constitutes a complex of different polysaccharide polymers, such as cellulose, hemicellulose and pectin and the phenolic polymer of lignin (Fincher, 2009; Weng et al., 2008). The amounts of these polymers, proportions between them, monomer compositions, depend on many factors and vary between species, organs, tissues, and cell types (McDougall, 1993). Cellulose covers about 70% of fiber weight, whereas hemicelluloses constitute approximately 15%. The rest are lignin (2–5%), pectin (1–15%), phenolic acids (0.1%), waxes, and inorganic compounds (2–5%) (Gorshkova et al., 1996; Morvan et al., 2003).

Cellulose is an unbranched biopolymer of β-1,4-glucose, and is present in two conformations, amorphous and crystalline, which determines physical properties of fiber. The less is the crystalline cellulose, the better is the water uptake (Bos et al., 2002; Stamboulis et al., 1999). The number of free —OH groups correlates with the hydrophilic character of cellulose (Bledzki et al., 2008). Pectin is a sugar polymer, mainly built from galacturonic acid backbone polymer, which is branched *via* α-1,4-glycoside bonds with various sugars (Baley et al., 2012). Hemicellulose strongly binds to cellulose *via* a number of hydrogen bonds. Apart from —OH groups,

Abbreviations: PHB, polyhydroxybutyrate; UPLC–MS, ultra performance liquid chromatography–mass spectroscopy; PDA, photodiode array; QTOF, quadrupole-time-of-flight; ESI+, positive electrospray ionization; BEH, bridged ethylene hybrid; DPPH, 1,1-diphenyl-2-picrylhydrazyl; CBD, cannabidiol; YPD, yeast extract-peptone-dextrose; MTT, 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

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hemicellulose contains a number of acetyl groups making it partially soluble in water and hygroscopic. The cellulose–hemicellulose structure is reinforced by lignin, which is covalently bonded to hemicelluloses. Lignin fiber component is synthesized within phenylpropanoid pathway and it is a kind of reservoir of phenolics for fiber. The presence and functionality of phenylpropanoid pathway in flax ensures the presence of other, different from the lignin and its precursors, phenylpropanoid compounds in flax. They are a wide and important group of secondary metabolites, involved in plant growth, development, and plant defense against pathogens (Cushnie and Lamb, 2005; Lattanzio et al., 2006; Winkel-Shirley, 2001). What distinguishes flax fiber from other natural fibers is that they are biologically active, due to the phenylpropanoid compounds.

In recent decades, studies on flax genetics rapidly developed giving the scientists and breeders tools for improved plant/fiber quality production (Cobb et al., 2013; Moose and Mumm, 2008; Wang et al., 2013). The most exploited method in modern plant biotechnology is generation of transgenic organisms for both, scientific and commercial purposes. Application of genetic engineering in basic and applied flax breeding research is now possible, because gene technologies and transformation techniques have been improved. It is expected that better understanding of the genes involved in fiber productivity, quality, and novel properties will provide targets for fiber quality improvements by genetic engineering methods leading to more diverse products derived from flax fibers. It is possible that improved knowledge on the development of cell wall will help to generate novel plant fibers, with better qualities (longer, thinner, soft, resilient, *etc.*), for textile or composite applications. Moreover, modification in plant cell wall will allow obtaining easily extractable fibers. One of such modifications may be introducing polyhydroxybutyrate (PHB) synthesis genes into the flax genome. We have previously generated transgenic flax plants bearing three bacterial genes from *Ralstonia eutropha* coding for PHB biosynthesis enzymes. Stem/fiber specific promoter from *16R* isoform of *14-3-3* gene (Aksamit et al., 2005) and the sequence of *Rubisco* gene responsible for transition to plastids have been used. The plants displayed unchanged phenotype and production of reasonable PHB quantities (Wróbel et al., 2004). This modification resulted also in fibers with much improved biomechanical properties, in which PHB is bound to cellulose in polymer of fibers by hydrogen and ester bonds during plant growth. Previously, those fibers were successfully used in the preparations of composites (Szopa et al., 2009) with polypropylene and polylactide matrices, which successfully served as tissue engineering scaffolds (Gredes et al., 2010). The metabolomic analysis by GS–MS of the *in vitro* grown plants revealed that genetic modification resulted in altered phenylpropanoid levels (Wróbel et al., 2004).

In the present research, the phenolic contents of flax fiber is studied in more detail in order to determine the impact of the modification on phenylpropanoid metabolism and establish the usefulness of flax products from PHB-overexpressing plants for biomedical applications, particularly wound dressing production. Chronic non-healing ulcers are a critical problem in clinical practice and are one of the major challenges for modern medicine. One of the problems in chronic wound pathogenesis is the poor cell proliferation in a wound bed linked to reactive oxygen species and inhibition of cell proliferation. In this regard, PHB is of special interest because in contact with body fluid it degrades to release D,L-β-hydroxybutyrate promoting proliferation of cells in high-density cultures thereby preventing apoptotic cell death (Cheng et al., 2006; Ji et al., 2008). This is the first report on the successful usage of PHB–cellulose composite fiber-based wound dressings to heal human chronic wounds of venous etiology.

2. Materials and methods

2.1. Plant material and growth conditions

Flax (var. Nike) seeds were provided by the Institute of Natural Fibers of Poland (Poznan, Poland). The seeds were germinated and grown in the greenhouse under the following conditions: 16 h at 21 °C in light, 8 h at 16 °C in darkness. The plants were grown both in soil (separate pots, watered each day) and in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with sucrose (1%) and agar (0.8%). Seeds obtained from the soil grown culture were used for field cultivation of flax plants, which further served as the source of fiber for fabric production.

2.2. Transgenic plant construction and selection

Transgenic flax type M plants bearing three genes of PHB synthesis from *R. eutropha* were generated and selected as described previously (Wróbel et al., 2004).

2.3. Flax retting

All transgenic plants were grown in fields near Wroclaw and harvested after 4 months. The collected plants were retted by spreading them out in the field for 6 weeks with turning the straw every 14 days. This is called “dew method” in which the microorganisms digest the plants cell wall polysaccharides during the retting period (Henriksson et al., 1997).

2.4. IR spectroscopy

Infrared (IR) spectra were detected and recorded in range of 400–4000 cm^{-1} (with resolution of 2 cm^{-1}) with an FT-IR Biorad 575C spectrometer (USA). Each sample was prepared with potassium bromide pellet and Nujol suspension. The process was performed at room temperature (20 °C).

2.5. Analysis of cellulose level

The cellulose content in fibers from control and transgenic plants was determined using colorimetric method (Updegraff, 1969). The first step was the removal of lignin, hemicelluloses and xylosans by incubation with a mixture of nitric and acetic acid (1:8, v/v, 30 min, 100 °C) and centrifugation. After two washes with pure water, the obtained pellet was suspended in 1 ml of 67% (v/v) sulfuric acid. The samples were diluted 10 times with anthrone reagent, and incubated for 15 min at 100 °C. Cellulose levels were measured spectrophotometrically at 620 nm with anthrone reagent as the blank.

2.6. Analysis of lignin level

To determine the level of lignin in the fibers, acetyl bromide method was used (Iiyama and Wallis, 1990). The fiber samples were obtained from the field-grown flax. The dried fibers were poured with 10 ml of water and incubated at 65 °C for 1 h with stirring every 10 min. In the next step, the samples were filtered through GF/A glass fiber filter and washed with four solvents: water, ethanol, acetone and diethyl ether. The filters were incubated at 70 °C for 12 h in glass vials. Then, the filters were incubated for 2 h at 50 °C with 2.5 ml of 25% acetyl bromide and after cooling mixed with 10 ml of 2 M NaOH and 12 ml of acetic acid. On the next day, the samples were analyzed spectrophotometrically at 280 nm with calibration curve prepared with coniferyl alcohol. The results are presented as coniferyl alcohol equivalents.

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2.7. Analysis of pectin level

A modification of the method described by Melton and Smith (2001) was used to determine pectin level in the fibers. Firstly, the samples were washed with 96% ethanol at 100 °C and centrifuged. Then, the pellet was treated with 80% ethanol at 80 °C, subsequently washed with a mixture of chloroform and methanol (1:1, v/v), and then with acetone. The resulting pellet was dried at 37 °C, frozen, and weighed. The level of total pectin was determined after acidic hydrolysis (100 μl of H_2SO_4 per 5 mg of the pellet). Then, 900 μl of distilled water were added in three steps (50 μl, 150 μl, and 700 μl) each followed by stirring (5 min, 4 °C). The pectin level was determined spectrophotometrically at 520 nm, and the calibration curve was prepared with galacturonic acid.

2.8. Phenolic component extraction

To extract phenolic compounds from fibers or fabric, a modified method described by Subba Rao and Muralikrishna was used (Subba Rao and Muralikrishna, 2002). Each sample (1 g) was ground using a Retsch mill and extracted with methanol thrice. Fractions were pooled, evaporated under vacuum, and resuspended in 1 ml of methanol. The remainders after the methanol extraction were used for analysis of cell wall-bound phenolic components. The pellet was hydrolyzed with 2 M NaOH at 37 °C. After 24 h, the pellet was discarded, and pH of the supernatants was adjusted to 3. The solutions were extracted with ethyl acetate thrice, fractions were combined and the organic solvent was dried under vacuum. The remainder was resuspended in 1 ml of methanol and used for further analysis.

2.9. Estimation of total phenolics

To determine the content of total free and ester bound phenolics the Folin–Ciocalteu method was used (Gómez-Alonso et al., 2002). To an aliquot of the extract, diluted Folin–Ciocalteu reagent was added. Then, to each sample, saturated sodium carbonate and water were added. Total phenolic content was measured spectrophotometrically at 725 nm. The results are presented as gallic acid equivalents.

2.10. UPLC analysis of phenolics

The flax fabric extracts were analyzed using BEH C18, 2.1 mm × 100 mm, 1.7 μm column on a Waters Acquity UPLC system equipped with a 2996 PDA detector and QTOF mass detector. The mobile phase was A = acetonitrile/B = 0.1% formic acid in a gradient flow: 1 min – 95% A and 5% B, 2–12 min – gradient to 70% A and 30% B, 12–15 min – gradient to 0% A and 100% B, and 15–17 min – gradient to 95% A and 5% B with a 0.4 ml/min flow rate. The mass spectra were acquired in ESI+ mode for 17 min in the range of 50–800 Da, under the following parameters: nitrogen flow 800 l/h, source temperature 70 °C, desolvation temperature cone 400 °C, capillary voltage 3.50, sampling cone 30, cone voltage ramp 40–60 V, scan time 0.2 s. The components were identified on the basis of retention times, ultraviolet spectra, as well as mass spectra of standard chemical compounds.

2.11. PHB determination

Fibers (30 g) were extracted trice with 0.5 l of chloroform. After the solvent was dried under vacuum, concentrated H_2SO_4 was used to hydrolyze the extracted PHB for 30 min at 90 °C. Samples were diluted 50× and analyzed using BEH C18, 2.1 mm × 100 mm, 1.7 mm column on a Waters Acquity UPLC system equipped with a 2996 PDA detector and QTOF mass detector. The mobile phase was A = acetonitrile/B = 0.1% formic acid in a gradient flow: 1 min, 1%/99% A/B, 2–5 min gradient to 40%/60% A/B, and 6–7 min gradient from 40% to100% A with a 0.4 ml/min flow rate.

2.12. Determination of antioxidant potential

Aliquots of 6 μl of the studied plant extracts were mixed with 200 μl of DPPH reagent (1,1-diphenyl-2-picrylhydrazyl). The samples were incubated at room temperature in darkness for 15 min, and then absorbance was measured at 515 nm. The control sample was 1 ml DPPH and 6 μl methanol. The blank sample was pure methanol. The antioxidative properties were expressed as antioxidative potential (equal to the inhibition of the free-radical reaction expressed as a percentage) (Brand-Williams et al., 1995).

2.13. Extraction of hydrophobic components

The fabrics were ground using Retsch mill and extracted with chloroform three times, the solvent was dried out and the remainder was resuspended in 100 μl methanol. After filtration through 0.25 μm Acrodisc, the extracts were used for further analyses.

2.14. UPLC analysis of hydrophobic components

Hydrophobic component analysis was performed with a Waters Acquity UPLC system equipped with a photodiode array detector using BEH C18 column (2.1 mm × 150 mm, 1.7 μm particles) heated to 40 °C. The mobile phase was A: acetonitrile, B: water in a gradient flow. 1 min 70% A/30% B, 2–5 min gradient to 100% A/0% B sustained for next 5 min and 10–11 min gradient to 70% A/30% B sustained till 12th min. The flow rate was 0.4 ml/min. Trifluoroacetic acid (0.05%) was added to A and B solvents to prevent from tailing. The photodiode array detector (PDA) was used to measure absorption in range of 210–500 nm. The detection and integration of the peaks was performed for cannabidiol (CBD) at 230 nm and for lutein at 445 nm.

2.15. Fabric preparation

The yarn was prepared in two ways. The first one employed traditional and industrial treatment methods involving several steps of boiling and bleaching with various chemicals, including alkali (NaOH) treatment resulting in white, soft yarn. The second one was based on chemically unprocessed yarn used subsequently for fabric production. The fabric was submitted to tumbling process that was similar to that used by the textile industry. The fabric obtained by this method had natural fiber color (gray) and was satisfactorily soft (similar to the softness obtained by traditional methods). For production of both fabrics, standard weaving method was used for fabric preparation. Warp and weft had the density of 65/dm and 85/dm, respectively, and the linear mass of 140TEX. The density of final flax fabric was 220 g/m^2.

2.16. Bacterial strains

Twelve clinical strains: *Staphylococcus aureus* ($n = 4$), *Staphylococcus epidermidis* ($n = 4$), and *E. faecalis* ($n = 4$) were used for determining antibacterial activity of transgenic fabric from genetically modified flax. The *S. aureus* ATCC 6538 strain from the American Type Culture Collection were used as references. The bacteria were stored at −70 °C in Trypticase Soy Broth with addition of 20% glycerol until further use.

2.17. Determination of antibacterial activity of fabric made from transgenic fibers

The antimicrobial activity of transgenic fabric was compared with that of cotton and non-transgenic by determining

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their influence on bacterial growth. The antimicrobial activity was tested by a method according to the ISO/DIS 20645: 2004 standards (http://www.iso.org/iso/home/store/catalogue_tc/catalogue_detail.htm?csnumber=35499). The sterilized specimens were placed on two-layer agar plates. Bacterial strains were inoculated onto the upper layer of the agar plates and incubated at 37 °C for 18 h. The level of antimicrobial activity of tested fabrics was then determined by examining inhibition of bacterial growth in the contact zone between sample and agar surface. Each assay was done in triplicate.

2.18. Pathogenic fungi strains

Six clinical strains of pathogenic fungi were used for determining antifungal activity of transgenic fabric made from genetically modified flax fibers: *Candida krusei* 153, *C. krusei* 264, *Cryptococcus neoformans* 2110, *Trichosporon cutanem* 662, *Candida tropicalis* 252, *Candida albicans* 10231. These strains were obtained from University of Wroclaw Microbiology Institute collection, Poland.

2.19. Determination of antifungal activity of fabric made from transgenic fibers

The fungi pre-cultured on YPD medium were diluted and spread on YPD agar plates, and tested fabrics were placed on the agar surface. Alternatively, the inoculum was resuspended in medium containing agar, poured onto plate, and a piece of fabric was embedded in the medium before it set. The plates were incubated at 37 °C for 24 h, then fungal colonies on the surface and "deep grown" (embedded fabric) was counted.

2.20. Determination of fibroblast viability by MTT test

Normal Human Dermal Fibroblasts (NHDF) (Lonza, Switzerland) cells were grown in 24-well plate at 50,000 cells per well in DMEM medium (Lonza, Switzerland) containing 1.5 g/l glucose, 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin and left for overnight adhesion. The medium was replaced with a fresh one after a day, and 0.5 cm^2 fragments of flax fabrics (sterilized by autoclaving beforehand) were put in the wells. Non-treated cells constituted the control. Plates were incubated for 24 h. Thereafter, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma, USA) solution was added in each well with final concentration of 0.25 mg/ml. Plates were incubated for 4 h and medium was discarded and replaced by 500 μl DMSO to dissolve formazan crystals and left for 30 min on the bench. Subsequently, absorbance values of the obtained solutions were recorded at 570 nm using an Asys UVM340 Microplate Reader (Biochrom, UK). The MTT assay was conducted three times to ensure the repeatability of results, which were presented in percentage of the control (100%).

2.21. Pre-clinical trial

2.21.1. Preparation of the linen dressing

Flax fabric was sterilized by autoclaving at 120 °C for 20 min. The size of the fabric for wound treatment was 10 cm × 10 cm. Each piece of the fabric was soaked with 0.9% NaCl solution prior to use.

2.21.2. Patients

The study group comprised of patients suffering from chronic non-healing venous ulcers. The patients were treated in the Dermatology out-patient clinic of the Ars Medica clinic in Wroclaw, Poland. The study obtained the approval of an independent bioethics committee. All the patients were informed regarding the purpose and course of the study. The wounds were all located on patients' legs. Twenty-two patients (11 female, 11 male, mean age 65 ± 8 years) were provided treatment for wound healing using dressings prepared from M fibers. The treatment with flax fabrics was directed to patients, whose local and general treatment had neither resulted in healing nor any inhibition in the progression of the ulceration. The study included only the patients whose wounds had lasted for at least 12 months. No case of macroscopic and microscopic bacterial infection in wound bed was reported.

2.21.3. Patient treatment

All patients participating in the study were first examined to assess their general health conditions and to rule out the presence of internal diseases that might influence the results. The patients were instructed on how to replace the flax dressing daily. The therapy lasted four weeks and during that time period the physician examined the wounds, measured their areas and interviewed the patients four times. The first flax dressing was applied by a nurse.

2.21.4. Measure of wound size

Wounds were photographed using fixed focal length lens to ensure the same point of view and scale. To have a correct scale indicator, commercially available industrial photographic color standard table was photographed along with the wounds. It was used both to set correct colors of the image and to measure the wound. When wound was too large to fit in a single image, several individual images were taken around the wound to have a good view for wound surface measurement. Wound measurements were conducted using photographic software by counting pixels within the wound borders. As a reference, the number of pixels per 1 cm^2 was used. The 1 cm^2 square was photographed for each image. If the wound was quite large to fit in a single picture, all fragments in successive pictures were summed.

2.22. Statistical analysis

Student's *t*-test was employed for statistical analyses of the results. The analyses were performed using Statistica 7 software (Statsoft, USA).

3. Results

3.1. The analysis of the fiber constituents

After four months of growth in the field, flax plants were harvested, and then after retting, fibers were released from the stems and industrially processed into yarn and further fabric. As the chemical composition determines quality of the fibers and its suitability for various applications (Morrison Iii et al., 2000; Morrison and Archibald, 1998), the fibers obtained through the standard retting process were analyzed and compared with fibers obtained from non-transgenic plants. The levels of polymers (cellulose, pectin, lignin), and phenolic components in flax fibers were measured and the data are presented in Table 1. The M fiber differs from its control counterpart in having PHB polymer and increased level of soluble phenolics (123% of the control) and ester bound phenolics (116.7% of control). The PHB level estimated after conversion to crotonic acid was 51 μg per 1 g of fiber, confirming the results obtained previously for the plants in *in vitro* culture (Wróbel et al., 2004). Since phenolic compounds are strong antioxidants, their increased level is promising for the biomedical application of the M fiber. The knowledge on fiber constituents and their quantity was mainly derived from chemical methods. Although the method yields satisfying results, it is destructive and does not provide any information on the spatial arrangement of these constituents. IR spectroscopy as a non-invasive method has been used previously to characterize flax fiber at the molecular level. Particular bands

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Table 1
The chemical composition of fibers from control and transgenic M line. The presented data are means of three repetitions ± standard deviations.

	Cellulose (μg/mg)	Pectin (μg/mg)	Lignin (μg/mg)	Soluble phenolics (ng/mg)	Ester bound phenolics (ng/mg)	PHB (μg/g)
Control	720 ± 20	40 ± 0.1	32.14 ± 0.4	65 ± 2	300 ± 5	0 ± 0
M	690 ± 60	38 ± 0.5	30.5 ± 0.6	80 ± 2	350 ± 8	51 ± 0.1

PHB – polyhydroxybutyrata. The results are statistically significant at $p < 0.05$.

of vibrational spectra were analyzed and correlated with the specific functional groups of cellulose, pectin and lignin. There were no differences in the component wavenumbers, but there were in the intensities of bands in control and M fiber. The increase in the intensity of contour in M fiber suggests higher crystalline structure of cellulose (Szopa et al., 2009).

3.2. Fabric preparation

Comparison of biochemical properties of fibers from transgenic and the control plants suggests that the M fibers should be a suitable material for wound dressing production. Traditionally, in order to obtain soft, white fabric, chemical and physical treatments are used. As it turned out, the industrial treatments dramatically decrease the lignin content by over 30% (42.28 ± 0.4 to 29.1 ± 0.6 μg/mg DW) and phenolic derivatives by over 20% (from 1.85 ± 0.11 to 1.26 ± 0.08 μg/mg DW). Moreover, the incubation of fibroblasts with processed (bleached) fabric resulted in immediate cell death (Fig. 1). Therefore, for biomedical application a new technology was developed for fabric preparation. The obtained textile (labeled non-processed M) was then analyzed for the content of biopolymers (cellulose, pectin, lignin), antioxidative/anti-inflammatory components and fabric biological effect, and the results were compared with the traditionally processed fabric. In contrast to the chemically processed M transgenic fabric, as well as non-processed non-transgenic control fabric, the non-processed M fabric demonstrated no cytotoxicity against cultured fibroblasts. The chemically processed fabric was noted to cause a dramatic decrease in fibroblast viability, thus it was highly toxic. Opposite effect was observed for the non-processed M fabric, which caused increased proliferation of the cells. The number of cells was higher by 36% compared to the control (untreated fibroblasts) after 24 h of incubation (Fig. 1). After longer incubation times (48 h, 72 h) similar results were observed.

3.3. Antioxidant contents in fabrics

In order to establish the identity of major phenolic components in flax fabric, UPLC analysis of methanol extractable (free phenolics) and NaOH released (bound phenolics) components was performed. Methanol extracts from flax fabrics were scanty of phenolic derivatives that could be determined in UPLC analysis. The most abundant component among free phenolics was a very hydrophilic component with maximum absorption at 308 nm (data not shown). The spectrum of this component was identical with *p*-coumaric acid, but the retention time suggested it was more hydrophilic (perhaps oligosaccharide) derivative of the acid. The component content was higher in M transgenic fiber compared to control fibers. The other components, like vanillin, ferulic and coumaric acids, were present in limited quantities (data not shown). The methanol extracted fabric was subsequently hydrolyzed in 2 M NaOH in order to release the esterified phenolics (Lozovaya et al., 1999). The UPLC–MS analysis of the NaOH hydrolysate revealed presence of several components: 4-hydroxybenzoic acid, vanillic acid, vanillin, *p*-coumaric acid, syringaldehyde, acetovanillone, and ferulic acid, however some of those components were found at very low concentration (Table 2).

The fabric produced from the transgenic flax fiber contained higher content of the assayed compounds. Vanillin content was found to be the most abundant phenolic in the flax fabric. It reached

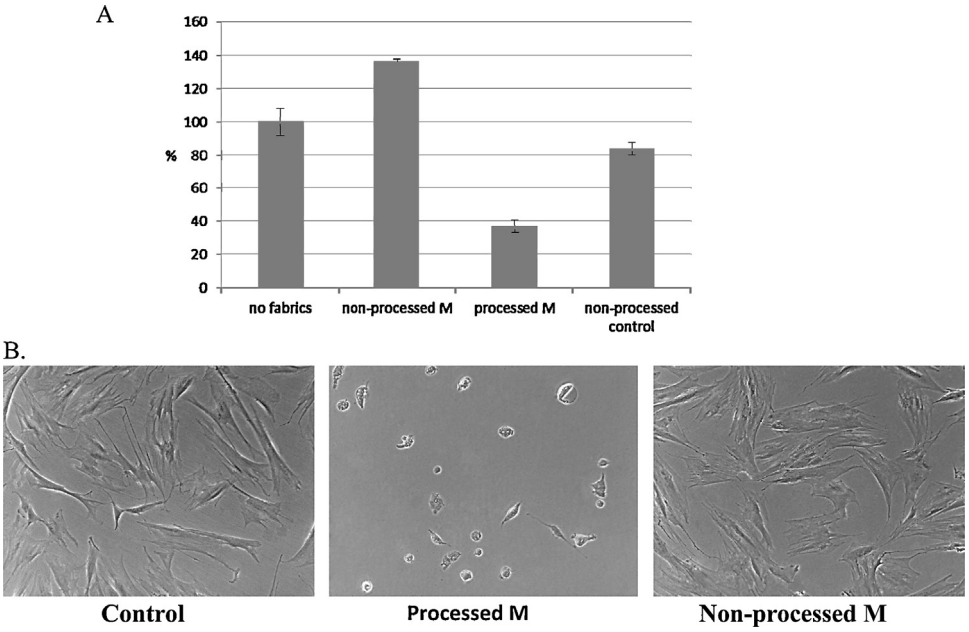

Fig. 1. Influence of fabric processing on cell viability. (A) Test of viability of fibroblasts cultured for 24 h in the presence of fabric made of processed and non-processed M transgenic flax fiber, and non-processed non-transgenic control flax fiber. The control (100%) is the value obtained for fibroblasts grown without fabric. The results are statistically significant at $p < 0.05$. (B) Microscope images of human normal fibroblast cells (control) and incubated for 24 h with fabric made using the standard industrial procedure (processed M), and a fabric obtained using the new method (non-processed M).

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Table 2
Compounds determined in fabrics made from control and transgenic M flax with the UPLC method. The presented data are means of three repetitions ± standard deviations.

	Ester-bound phenolics						CAL (ng/mg)	LU (ng/mg)
	HBE (μg/g)	VA (μg/g)	VE (μg/g)	COU (μg/g)	SYE (μg/g)	FE (μg/g)		
Control	3.51 ± 0.03	20.16 ± 0.21	52.25 ± 0.85	7.95 ± 0.19	22.23 ± 0.25	4.98 ± 0.10	4.88 ± 0.40	0.6 ± 0.06
M	3.48 ± 0.02*	22.13 ± 0.45*	58.26 ± 0.38*	8.21 ± 0.17*	25.98 ± 0.36*	5.11 ± 0.01	5.03 ± 0.28	4.01 ± 0.66*

4-Hydroxybenzoic acid (HBE), vanillic acid (VA), vanilline (VE), p-coumaric acid (COU), syringaldehyde (SYE), ferulic acid (FE), cannabidiol (CAL), and lutein (LU).
* Statistically significant at $p < 0.05$.

Table 3
Inhibition of the bacterial growth by fabric made from the non-transgenic control and transgenic M flax; "+" normal growth, "–" inhibition.

Strains	Cotton	Control	M
S. aureus ATCC 6538	–	–	–
S. aureus 1	+	+	–
S. aureus 2	+	+	–
S. aureus 3	–	–	–
S. aureus 4	–	–	–
S. epidermidis 1	+	+	+
S. epidermidis 2	+	–	–
S. epidermidis 3	–	–	–
S. epidermidis 4	–	–	–
E. faecalis 1	+	+	+
E. faecalis 2	+	+	+
E. faecalis 3	+	+	+
E. faecalis 4	+	+	+

almost 60 μg/g in the transgenic fabric M and was over 10% higher when compared to the control. The highest increase (over 15%) in comparison to the control was observed for syringaldehyde. Total phenolic compound content in the transgenic fabric was 10% higher than that in the control.

In our recent work we found two hydrophobic terpenoid components in flax fabric at reasonable quantities. First was lutein, a strong lipophilic antioxidant, belonging to carotenoid family, which might contribute to anti-inflammatory properties of flax fibers (Styrczewska et al., 2013). This component was found in all flax fabrics, however, as determined by UPLC analysis its level was highly elevated in M fibers when compared to control. The second and new component, based on mass spectrometry analysis, ultraviolet spectrum, and retention time in UPLC was identified as a terpenophenolic component – CBD. Further *in vitro* analysis of flax CBD effect on human fibroblast revealed its anti-inflammatory properties (Styrczewska et al., 2012). The compound was found to be present in all tested fabrics. The CBD level was slightly elevated in M fibers.

The presence of phenylpropanoid and isoprenoid derivatives in fabric strongly suggests its antioxidant potential. Thus fabric extracts were used for antioxidative properties determination with use of DPPH as a stable free radical. It was observed that fabric from transgenic M line showed higher percentage of free radical quenching (47.83%) in comparison to the control (43.17%).

3.4. Antimicrobial activity

3.4.1. Antibacterial activity

Antibacterial activity of flax fabrics prepared from control and M fibers was tested, as one of the major problems in a clinical practice is the wound infection. In this case, materials which display antimicrobial activity or at least do not promote microbial growth are preferable. The M fabric was tested against twelve clinical bacterial strains (*S. aureus, S. epidermidis* and *E. faecalis*). For comparison, cotton fabric being almost pure cellulose was included into this experiment. The results can be found in Table 3. The main difference between the M and control fabric was the difference in growth rate of *S. aureus* isolates. A higher antibacterial activity was observed for M fabric when compared to that of the control. Clinical strains of *S. epidermidis* were susceptible to both of the flax fabrics except for *S. epidermidis 1*, which was resistant to all tested materials. Both of the tested fabrics exhibited no inhibitory effect against *E. faecalis*. All fabric samples inhibited growth of bacteria only on the contact surface.

Table 4
The impact of the control and transgenic M flax fabric embedded in pathogenic fungal growth medium. The number of "+" indicates intensity of fungal growth, "–" complete inhibition, total lack of colonies.

Strain	Fabric	
	Control flax	M flax
Candida krusei 264	–	–
Cryptococcus neoformans 2110	+++	+
Trichosporon cutanem 662	++++++	+++
Candida tropicalis 252	++++	++
Candida albicans 10231	++++	++

3.4.2. Antifungal activity

The antifungal properties of flax fabrics were tested using several clinical strains of pathogenic fungi. Three different methods were used – testing in a liquid medium when fabric fragments were added to culture medium and two tests on agar solidified plates – one with the fabric placed on a surface, and another with the fabric embedded in a medium. Antifungal activity of fabrics was not detected when assayed in liquid medium (data not shown), however, the surface inhibition of fungal growth on flax fabric was observed. Fig. 2 shows fungal growth on cotton (included in experiment for comparison) as well as non-transgenic flax and transgenic fabric. It is clearly visible that fungal colonies form easily on the surface of cotton and at the same time almost no fungal growth was observed on the flax fabrics. No major differences between both flax fabrics (the control and M type) were observed. However, this kind of test is difficult to evaluate due to uneven distribution of yeast colonies on a plate. For this reason, the test was repeated with fabric embedded in the agar-solidified medium to which a fungal inoculum was added before pouring the plates. In this test, the differences were observed in a number of deep-grown colonies. The number of deep-grown colonies was visibly smaller in area over the fabric, and moreover the fabric made from transgenic flax shown a higher degree of inhibition (Table 4). The transgenic M fabric was most effective against *Cryptococcus neoformans*. In general, the tested flax fabrics showed promising antifungal properties. It can be speculated that the level of antimicrobial components is quite low in a fabric, and thus they are diluted in a large volume of a liquid medium, but these compounds are released in sufficient quantities to the immediate area to inhibit fungal growth in a close proximity.

3.5. Chronic wound treatment – pre-clinical trial

Since positive effect on cell proliferation and no cytotoxicity against cultured fibroblasts (Fig. 1) were observed and allergy test on animals proved to be negative (data not shown), the fabric from transgenic M fibers might be used for wound dressing preparing

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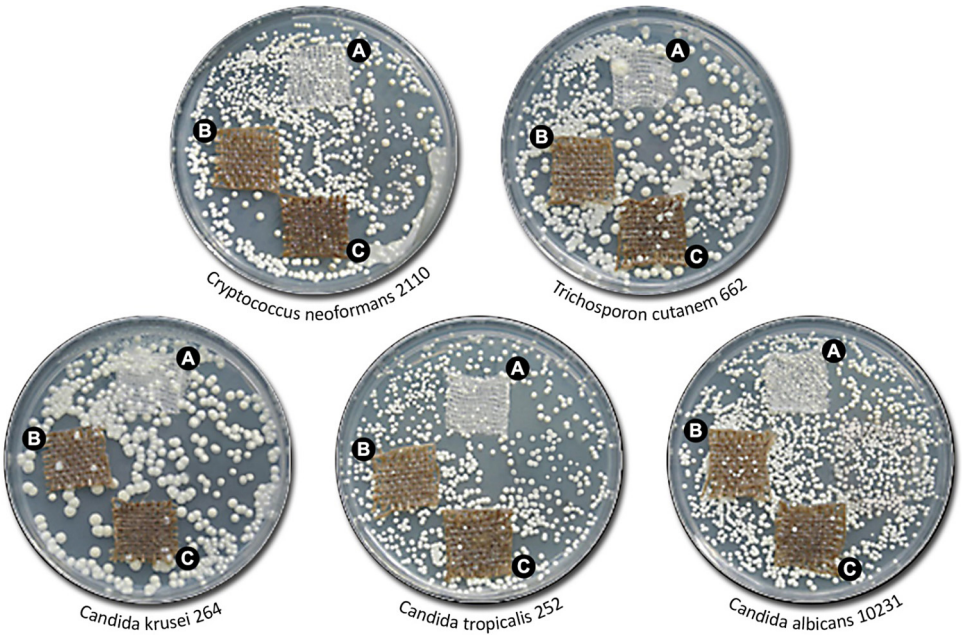

Fig. 2. Surface inhibition of growth of fungal colonies on cotton fabric (A), flax fabric – M type (B), and the control (C).

and testing on patients. To describe the changes in wounds after flax dressing treatments, single parameter with most objective character was considered and this was wound size.

After four-week treatment, the wound size turned out to be significantly reduced (Fig. 3A) and the mean decrease in the size was 28% (6 cm^2). The statistical analysis (*T* test for dependent samples) showed that M dressings were more effective in reducing wound size than cotton dressings with a statistical significance ($p < 0.001$). Fig. 3B shows an example of wound healing process under the action of M dressings. Already after a week of treatment, visible epidermisation occurred. The level of wound exudation was decreased and the patients reported on diminished level of pain sensation during M dressing usage.

4. Discussion

Since the beginning of agriculture, breeders selected plants with the best possible properties, taking into consideration their suitability for human needs. In flax breeding those modifications include increased pathogen resistance, oil quality and improved properties of flax fibers. In the recent years, biotechnological approach was employed in order to adapt flax fibers for medicinal use. The first strategy was to generate flax plants overproducing flavonoid compounds by simultaneous expression of three *Petunia hybrida* genes (the W92 plants) (Lorenc-Kukula et al., 2005; Styrczewska et al., 2012). Subsequently, fibers from those plants were used for wound dressings which were tested for healing of chronic skin ulcerations. Secondly, those fibers served as a source of antimicrobial components (Czemplik et al., 2011; Skorkowska-Telichowska et al., 2010). Recently, the new flax fiber producing PHB has become of great interest. Polyhydroxybutyrate upon contact with body fluids degrades to monomers of D,L-β-hydroxybutyrate. The monomers prevent apoptosis in high-density cultures (Cheng et al., 2006), thus they present a perfect solution in applications where the regeneration of large numbers of cells are required. Polyhydroxybutyrate itself has been used for the production of surgical tools (pins, sutures, staples), in tissue engineering (blood vessel replacements, bone replacements and plates, implants, cardiovascular patches or nerve cuffs) (Misra et al., 2006; Stojkovic et al., 2013), and as micro-carriers in drug delivery systems (Shrivastav et al., 2013). The recent finding that PHB monomers activate global cell transcription (inhibition histone deacetylases class I) and cell proliferation by preventing cell death in high-density culture makes this biodegradable polymer attractive for chronic wound healing treatments (Cheng et al., 2006; Ji et al., 2008). Those PHB-containing flax fibers embedded in a polylactide matrices were successfully used as tissue engineering scaffolds in rats (Gredes et al., 2010). The tested biocomposites did not show any inflammation response after subcutaneous insertion and showed a good biocompatibility with the muscle tissue. In particular, the mRNA expression of vascular endothelial growth factor was unchanged when PHB composites were used in contrast to composites made from unmodified fibers. So it was concluded that introduction of new flax bandages from plants producing "bioplastic" fibers can provoke induction of cell proliferation, which is crucial in the healing process. When the fabric generated from the plants producing PHB was tested for their aptness for wound healing, a significant decrease in the wound size was detected. The reduction covered 96% subjects upon treatment with M dressing, which is 35% more than that in previously tested W92 fabric. In only one case the size of wound was very slightly (1.5%) increased when treated with M fabric, which is also far better result when compared with that of W92 dressing, where 9 subjects showed increase or no changes in terms of this parameter (Skorkowska-Telichowska et al., 2010). Thus, it is suggested that the fabric containing both, PHB and increased level of phenylpropanoid compounds, is of higher efficacy in wound treatment than the modified, flavonoids enriched, linseed fibers.

Those properties can be attributed to couple PHB content with the increased phenolic derivatives content in M fibers. Phenolics are known to be antioxidant, anti-inflammatory and antimicrobial agents, thus they improve bioactive function of flax fibers. First of all, they scavenge oxygen free radicals, which participate in chronic wound pathogenesis and hinder healing processes. Oxidative stress is responsible for hampering of many cellular mechanisms and leads to fibroblast apoptosis (Wlaschek and Scharffetter-Kochanek, 2005). Moreover, phenolic compounds act as chelators of metal ions, thus impede their actions in free radical generation. In addition, as phenolics modulate the level of oxidants, and influence the cellular signal transduction mediated by the latter (regulation of

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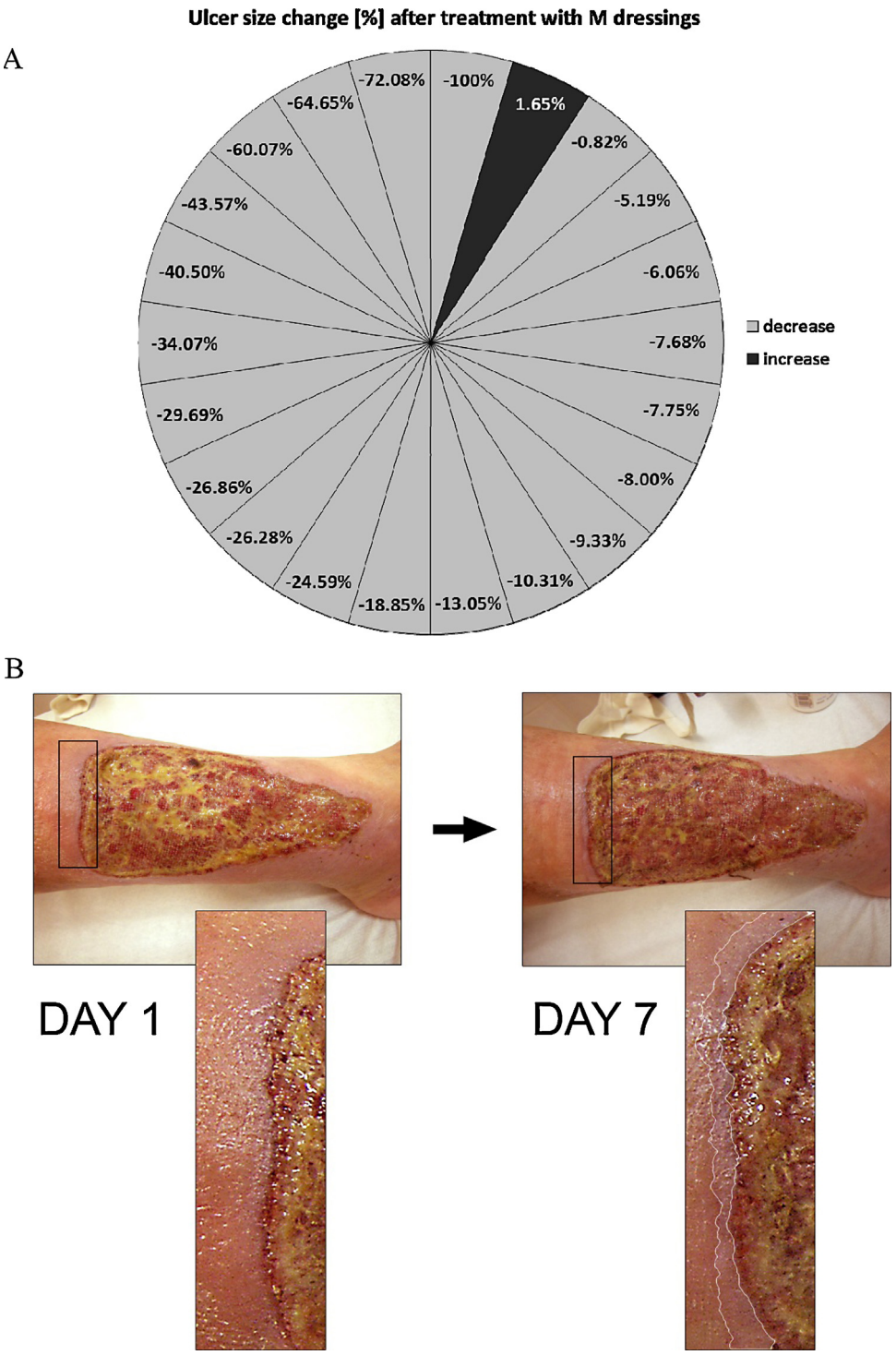

Fig. 3. The percentage of change in wound size after the treatment with M dressings. In 1 out of 22 patients (black color in the diagram), an increase in the ulcer size was observed. In one case, complete cicatrisation of the wound was observed (−100%) (A). The example of the chronic skin wound treatment with M dressing. The magnification shows decreased fibrin, enhanced granulation and a build-up of new dermal tissue (contoured white) only after a week of treatment (B).

cytokines and chemokines through NFκB leading to modulation of inflammatory response) (Amaral et al., 2009; Gomes et al., 2008; Rahman et al., 2006). It has been reported that low molecular antioxidants from the phenylpropanoid pathway promote healing of long-lasting wounds (Angerhofer and Giacomoni, 2008; Chiang et al., 2005; Phan et al., 2001). Increased phenolic content together with the presence of PHB make the new flax fiber an excellent material for the production of wound dressings. However, as was shown, the typical industrial processing of fiber and fabric production removes phenolic constituents and even made the product toxic for human cells. Thus in the case of medical device production, a more gentle method is necessary such as the one developed in our laboratory.

In addition, fibers isolated from field grown M plants were characterized by elevated biomechanical parameters (Young modulus, strength) (Wrobel-Kwiatkowska et al., 2009). Structurally the PHB-cellulose fibers are different from the control plants and the plants overproducing phenylpropanoid compounds (flax called

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W92) used previously for the preparation of wound dressing. The first showed more intra- and intermolecular hydrogen bonds in the polymer complex suggesting its higher crystalline structure. The intra-molecular hydrogen bonds give a significant stiffness to the cellulose molecules. The results of IR study led to the conclusion that cellulose matrix of transgenic W92 fibers is not as tightly structured as that from the M fibers, where the fibers of cellulose polymer are more closely bound (Szopa et al., 2009). The biopolymer complex arrangement can be influenced by lignin and pectin. The chemical and spectroscopic analysis confirmed that there are no major differences in these components between fibers from the transgenic M and control plants. However, difference in the cellulose content and arrangements has been detected. Higher level of cellulose polymer and higher numbers of intra- and intermolecular hydrogen bonds, and thus polymer crystallinity in fiber from M plants is strongly suggested, but at the moment it is difficult to speculate if this parameter can influence the wound healing.

An important issue during chronic wound treatment is the infection with pathogenic bacteria and fungi, which are related to patients' morbidity and contribute to the increase of health care costs. The M flax fabric inhibits the growth of pathogenic microorganisms and it is more effective than the control and cotton fabric. The inhibition of microorganism growth is important for wound protection against primary or secondary infection, especially in case of chronic ulceration. Very often, the infection is caused by antibiotic-resistant bacterial and fungal strains, and therefore new methods to overcome the infections are required. Antimicrobial activity of flax fabric originates from a mixture of factors, the structure and composition of biopolymers and phenylpropanoid content bound to the polymer being among them. Phenolics have antimicrobial properties (Cava-Roda et al., 2012; Stojkovic et al., 2013). In particular, ferulic acid was found to inhibit bacterial and fungal growth (Cushnie and Lamb, 2005; Papadopoulou et al., 2005; Pereira et al., 2007). Also CBD, which was found to be present in flax fibers, may participate in their antimicrobial activity (Appendino et al., 2008). However, as of yet, the antimicrobial nature of flax fiber is not fully recognized at the molecular level. Presumably, it is a combined action of many components found in flax fiber such as phenolics, terpenoids, sugars and fatty acids (Cowan, 1999).

To conclude, the unprocessed fabric based on flax plants producing PHB, is highly effective for wound dressing with a possible usage against chronic skin ulceration. Mild antibacterial and antifungal activity may help prevent any contamination during the healing process. In addition, since these fibrous plants produce high quantities of fiber (compared to previously used linseed fiber), the costs of wound treatment is considerably low, which is especially important in case of long-term therapy of chronic wounds.

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